UNIVERSITAT POLITÈCNICA DE CATALUNYA
BARCELONATECH
Escola Superior d'Agricultura de Barcelona

Genetic transformation of the bacterium *Bacillus firmus* for GFP expression and confocal laser microscopy analysis

Treball final de grau

Enginyeria de Sistemes Biològics

Autor: Daniel Beltrán Anadón

Tutores: Francesc Xavier Sorribas

Nuria Escudero

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Resum

Bacillus firmus I-1582 és un bacteri amb efectes nematicides en nematodes formadors d'agalles, un grup de paràsits de plantes que pertanyen al gènere *Meloidogyne*. Aquest bacteri és capaç de colonitzar la rizosfera de plantes d'alguns cultius econòmicament importants i protegir-los dels nematodes paràsits reduint la emergència dels juvenils. En aquest treball de fi de grau *B. firmus* va ser transformat amb un gen de la proteïna verd fluorescent. Per transformar el bacteri va ser necessària la prèvia formació de protoplasts utilitzant lisozima i la adició de polietilenglicol. El plasmidi que conté el gen, pAD43-25, va ser clonat i extret d'un cultiu d'*E. coli* ECE166. Un cop el plasmidi era estable, els transformants van ser inoculats a arrel de tomàquet i ous de nematodes. Les mostres van ser analitzades després de 48 hores a 35°C amb microscòpia làser confocal per observar la distribució dels bacteris i la formació de biofilm, el qual també era fluorescent. A les imatges presses s'aprecia que el bacteri no ha estat capaç de colonitzar l'arrel y que tampoc ha penetrat als ous. No obstant sí que sembla que alguns productes citoplasmàtics del bacteri poden travessar la paret de l'ou.

Resumen

Bacillus firmus I-1582 es una bacteria con efectos nematicidas en nematodos formadores de agallas, un grupo de parásitos de plantas pertenecientes al género *Meloidogyne*. Esta bacteria es capaz de colonizar la rizosfera de plantas de algunos cultivos económicamente importantes y protegerlos de los nematodos parásitos reduciendo la emergencia de juveniles, su única etapa infectiva. En este trabajo de fin de grado *B. firmus* fue transformado con un gen de la proteína verde fluorescente. Para transformar la bacteria fue necesaria la previa formación de protoplastos usando lisozima y la adición de polietilenglicol. El plásmido que contiene el gen, pAD43-25, fue clonado y extraído de un cultivo de *E. coli* ECE166. Una vez el plásmido era estable, los transformantes fueron inoculados en raíz de tomate y huevos de nematodos. Las muestras fueron analizadas después de 48 horas a 35°C con microscopía láser confocal para observar la distribución de las bacterias y la formación de biofilm, el cual también era fluorescente. En las imágenes tomadas se aprecia que la bacteria no ha sido capaz de colonizar la raíz y que tampoco ha penetrado en los huevos. No obstante sí que parece que algunos productos citoplasmáticos de la bacteria puedan atravesar la pared del huevo.

Abstract

Bacillus firmus I-1582 is a bacterium with nematicidal effects on root-knot nematodes, a group of plant parasites pertaining to the genus *Meloidogyne*. This bacterium is able to colonize the plant rhizosphere of some economically important crops and protect them from nematode parasites by reducing their juvenile hatching, their only infective stage. In this final degree project *B. firmus* was transformed with a green fluorescent protein gene. To transform the bacterium it was necessary the previous protoplast formation using lysozyme and the addition of polyethylene glycol. The plasmid containing this gene, pAD43-25, was cloned and extracted from an *E. coli* ECE166 culture. Once the plasmid was stable, the transformant was inoculated in tomato roots and nematode eggs. The samples were analyzed after 48 hours at 35°C with confocal laser microscopy to watch the distribution of the bacteria and the biofilm formation, which was fluorescent too. In the taken images is shown that the bacterium was not able to colonize the root neither penetrate the eggs. Although it seems that some of the bacterial cytoplasmic products can cross the egg wall.

Summary

Summary of figures and abbreviations

Figures

Abbreviations

- CI: Chloroform-Isoamyl alcohol
- DNA: Deoxyribonucleic Acid
- GFP: Green Fluorescent Protein
- LB: Luria-Bertani
- pb: Pair of Bases
- PCR: Polymerase Chain Reaction
- PEG: Polyethylene glycol
- upp: Uracil phosphoribosyltransferase

1. Introduction

Plant parasitic nematodes belong to *Meloidogyne* genus cause yield losses to some economically horticultural important crops such as tomato and cucumber. When a juvenile (J2), the only infectivity stage of the nematode, finds a host plant, it penetrates the root with the stylet and migrates towards the vascular cylinder where it induces the feeding site by redifferentation of five to seven cells into hypertrophied multinucleate cells known as giant cells which are the only source of nutrients for the remaining developmental stages of nematode, that is J3, J4 and the adult female. The symptoms of root infection are shown as galls or root-knots altering root capability to absorb water and nutrients. Then, the above ground plant symptoms are wilting, nutrient deficiencies, dwarfing, and damping-off, depending on the nematode densities at planting and the host status (Perry et al., 2009) [17].

Traditionally, plant parasitic nematodes have been controlled with nematicides. Nematicides have being evolving since 1950. Nowadays in the European Union there is a usage directive to reduce the use and dependence of old non-sustainable control methods approved in 2009 [6]. New biological nematicidal techniques reduce the risks for human health and the environment.

Bacillus firmus (see annexed B) is a gram positive bacterium. The strain I-1582 of *B. firmus* has shown nematicidal effects (Keren-Zur et al. 2000) [13], affecting egg hatching, inducing paralysis of motile stages, and mortality of various species of nematodes (Crow 2014 [5], Terefe et al. 2009 [18], Xiong et al. 2015 [24])[5]. It is also a probiotic organism that colonizes the plant rhizosphere, consumes root exudates and produces hormones that stimulate the root growth (Bayer 2014) [2]. Nowadays there are some biological nematicide formulations in the market based on this *B. firmus* isolate.

Some studies carried out by part of the UPC-Plant Pathology research group have shown that *B. firmus* strain I-1582 is able to induce plant defense mechanisms in split-root experiments reducing the number of eggs produced by root-knot nematodes in tomato and cucumber, is able to decompose egg shell and to degrade the embryo, to colonize cucumber roots and to develop onto the roots forming biofilms. The main objective of this project was to transform *B. firmus* by GFP to

characterize the bacterial-plant and the bacterial-nematode egg interactions at cellular level. The fluorescence allows a better observation through confocal laser microscopy of the bacteria and their biofilm formation. Studying the distribution of the bacterium in a colonized root or an infested egg will help to understand its behavior. This tool will be one more step to fully understand the protection mechanism that *B. firmus* induces in the host plant against *Meloidogyne*.

The GFP (Green fluorescent protein) is a widely used marker in molecular biology. It is easy to observe with fluorescent microscopy. This protein can be synthetized alone or attached to other proteins and it usually doesn't affect to the organisms metabolisms. Nowadays there are a lot of variants from the original GFP discovered in a jellyfish, *Aequorea Victoria*.

An example of what we are looking for would be the images shown in the Figure 1:

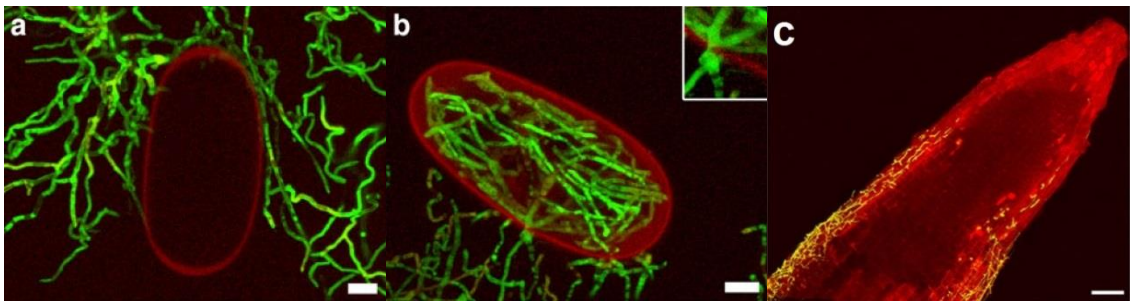

Figure 1: Images compilation from nematode eggs ("a" and "b") and tomato roots ("c") infested/colonized with a GFP transformant of *Pochonia chlamydosporia* (Escudero and Lopez-Llorca, 2012) [9]. At the image "a" an egg infested with the fungi 48 hours after the inoculation is shown. The egg in the image "b" had been inoculated 72 hours ago and in the up-right corner there is an augmented region of the photo where the organism crossed the egg wall. In both images the bar measures 10 µm. At the image "c" there is a tomato root also colonized by *Pochonia* and the bar measures 75 µm.

The plasmid containing the GFP, pAD43-25, was given from the Bacillus Genetic Stock Center and it was delivered inside an *E. coli* culture, ECE166. For this reason the previous extraction of the plasmid from *E. coli* was needed. Another previous step to the transformation was the protoplasts formation. Protoplasts are cells without wall. *B. firmus* has a natural wall which can be removed with lysozyme. This way the cell doesn't dies, because its membrane remains under the wall, but the cell gets more susceptible to be transformed.

2. Objectives

The main objectives of this final degree project were:

1) To extract and purify the plasmid pAD43-25 from an *E. coli* ECE166 culture.
2) To get *B. firmus* protoplasts.
3) To genetically transform the *Bacillus firmus* strain I-1582 with the GFP gene.
4) To verify the GFP expression in *B. firmus*.
5) To observe and analyze the bacterial root colonization and a nematode egg parasitism using confocal laser microscopy.

Other *Bacillus* species as *Bacillus subtilis* have also been transformed with GFP to observe their biofilm formation (Hamon and Lazazzera, 2001 [11]; Li et al., 2013 [14])

3. Materials and methods

3.1. Bacteria strains, plasmid and bacterial growing conditions

The two bacteria used in this study were *Bacillus firmus* (I-1582) and *Escherichia coli* (ECE166), provided from Bayer and Bacillus Genetic Stock Center respectively. Both bacteria were grown on Luria-Bertani medium (LB) at 35ºC. In case of selective medium two antibiotics were used: chloramphenicol (Sigma) (20 µg/ml) for *B. firmus* and ampicillin (Sigma) (100 µg/ml) for *E. coli*.

The plasmid used in the experiment was pAD43-25 (see annexed D) and it was contained in *E. coli* ECE166. It contains a GFP gene with a *Bacillus* promoter and 2 antibiotic resistance genes: ampicillin resistance for *E. coli* and chloramphenicol resistance for both *E. coli* and *B. firmus*.

3.2. Media and buffers

All media and buffers used during this final degree project are listed below:

- LB (1 L): 10 g peptone, 5 g yeast extract and 10 g NaCl. For solid medium, media was supplemented with 15 g of agar.
- 4X Pennasay (1 L): 6 g beef extract, 6 g yeast extract, 20 g peptone, 4 g glucose, 14 g NaCl, 14,72 g dipotassium phosphate and 5,28 g monopotassium phosphate.
- 2X SMM (1 L): 342 g sucrose, 4,64 g maleic acid and 8 ml $MgCl_2$ 2 M.
- SMMP: ½ volume of 4X Penassay and ½ volume of 2X SMM. pH was raised to 6,4 with NaOH 10 M. In this case, the medium was not autoclaved to avoid pH decrease so mediums and soda were sterile.
- 10X TBE buffer: 890 mM Tris-borate, 890 mM boric acid and 20 mM EDTA.
- Loading buffer 10X: 6,84 M Glicerol, 30 mM EDTA, Bromophenol blue 0.1 % and xylene cyanol 0.1 %.

- Lysis solution: 0.25 ml of Tris 1 M ph 8, 0.2 ml of EDTA 0.5 M, 0.17 ml of Sucrose 60 % and 2,38 ml of H_2O.
- Saline solution: 0.1 ml of NaOH 10 M, 0.5 ml of SDS 10 % and 4,4 ml of H_2O.
- CTAB Buffer: Tris 0.1 M pH 8.4, NaCl 1.4 M, EDTA 0.025 M pH 7.5, CTAB 2 % and PVP 2 %.
- TNE Buffer: Tris 10 mM. EDTA 1 mM, NaCl 100 mM.

3.3. Plasmid extraction from *E. coli*

After growing *E. coli* for 2 days on LB media supplemented with 100 µg/ml of ampicillin, 3 ml of culture were centrifuged at 8900 g for 1 minute. After that step, pellet was resuspended in 0.1 ml of lysis solution using a vortex. In the next step, 0.2 ml of saline solution were added and homogenized with the vortex. Bacteria were kept on ice for 10 minutes and shake with the vortex every 2 minutes. After the incubation step, 0.15 ml of sodium acetate 3 M was added and the cells were kept back on ice for 10 more minutes and shake every 5 minutes. After that, the mix was centrifuged at 8900 g and 4 °C for 10 minutes. The supernatant was collected, mixed with 1 ml of pure ethanol at -20 °C and centrifuged at 11000 g and 4 °C for 10 minutes. Then, after discard the supernatant, pellet was washed with 70 % ethanol at -20 °C and centrifuged at 11000 g at 4 °C for 5 minutes to remove all the alcohol. Finally, pellet was diluted in 30 µl of DNase free H_2O with 1 µl of RNase.

3.4. DNA plasmid purification

First an electrophoresis (see annexed C) with a 0.8 % agarose gel (0.8 g of agarose disolved in 100 ml of 0,5X TBE buffer) was run to pull apart the plasmid DNA from the genomic bacterial DNA. The agarose was dissolved heating the mixture in a microwave oven until it started to boil up. At this point, the mixture was taken out, shaken and heated again until boil it up. This process was repeated 3 or 4 times to fully dissolved the agarose in the buffer. After that, the suspension rested to reach a lower temperature. Before getting solidified, 2 µl of ethidium bromide were added and

the mix was spilled in the mold. Once the gel was polymerized it was placed in the bucket and covered by 0,5X TBE buffer. At this point, the gel is ready to be loaded.

The whole plasmid samples (≈31 µl) were loaded with 8 µl of loading buffer. After that step, the marker (2 µl of mass ruler [see annexed E], 2 µl of loading buffer and 6 µl of DNase free H_2O) and the negative control (2 µl of loading buffer and 8 µl of H_2O) were loaded.

After the electrophoresis was run the plasmid size (≈7000 pb) was identified thanks to the mass ruler. At this point the segments of gel containing the desired bars were cut by using a scalpel. To remove the agarose from the plasmid bar a commercial kit was used (NuceloSpin gel and PCR clean-up, see annexed E) according to the manufacturer's instructions.

3.5. Plasmid quantification

In order to know the DNA plasmid concentration after the extraction and purification an electrophoresis with a mass ladder was run. The mass ladder is a marker with a very accurate range of concentrations that allows us to make a calibration line to quantify the amount of DNA in our samples (see Annex E). In this case only were added a few microliters from each samples.

The agarose gel, the mass ruler and the negative control were prepared as described in section 3.4. The mass ladder was loaded with 2 µl of mass ladder itself, 2 µl of loading buffer and 6 µl of H_2O. The plasmid was loaded with 5 µl of plasmid, 2 µl of loading buffer and 3 µl of H_2O. After the electrophoresis, the gel was analyzed with a transilluminator (Gel Doc 2000 from Bio Rad) and with the software Quantity One. The photo is shown in the results and discussion section.

3.6. Preparation of protoplasts

Protoplasts were prepared using the protocol described by Ito et al., 1997 [12] with sight modification, for Bacillus a higher concentration of lysozyme (Sigma) was used. *B. firmus* was grown overnight in Penassay medium. After that step, 3 ml from this culture were harvested by centrifugation at 800 g and 4 °C for 10 minutes. The cells were resuspended in 0,1 ml SMMP medium and 44 mg of lysozyme were added and it was maintained at 37 °C with gentle shaking for 75 minutes. After that, cells were centrifuged at 56 g for 30 minutes. Pellet was washed twice and resuspended in 0,1 ml of SMMP medium. Protoplasts were used immediately for transformation.

*This amount of lysozyme was used due to experimental errors. Less lysozyme may be enough.

3.7. Transformation of *Bacillus firmus* with the plasmid pAD43-25

For this experiment a previous transformation protocol was adapted (Chang and Cohen, 1979) [4]. Briefly, 5 µl of plasmid (14 ng/µl) were mixed with 5 µl of 2X SMM medium and then 50 µl of protoplasts obtained as described in section 3.6 were added, followed by addition of 150 µl of 40 % PEG. Also a negative control was made without plasmid. After 2 minutes of incubation 0,5 ml of SMMP were added to the mixture and protoplasts were recovered by centrifugation at 560 g for 10 minutes. Pellet was resuspended in 0,1 ml of SMMP and kept at 30 °C with gentle shaking for 4 hours. Finally, the cells were plated in LB medium amended with ampicillin (100 µg/ml) and incubated at 37 °C for 2 days.

The colonies that grown in the LB selective media were observed in the fluorescence microscope located in the UPC to check that the transformation was right .

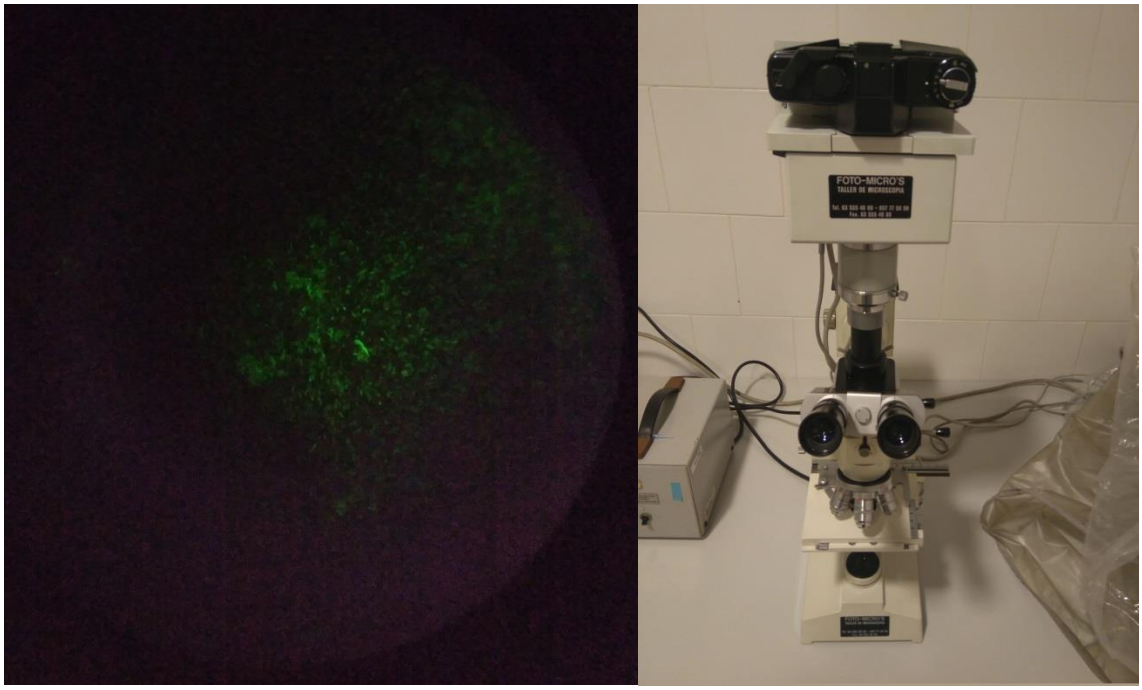

Figure 2: *Bacillus firmus* GFP-transformant observed with a fluorescence microscope at 400 augments and the microscope itself (Foto Micro's).

In order to verify that the transformed bacterium was *Bacillus firmus* and no other *Bacillus* from the environment, which also could express the GFP gene, a DNA extraction followed by a PCR (see annexed C) were done with the bacteria DNA from 10 random fluorescent colonies. This could seem a very improbable case but actually it's not. *Bacillus* is a ubiquitous genus in the nature and some species produce aerial spores (Turnbull and Kramer, 1991) [22] so the risk of contamination is pretty high.

3.8. DNA extraction from bacteria

10 random colonies verified by fluorescence microscopy were grown for 2 days in LB media amended by chloranfenicol (20 µg/ml). Transformants were streaked in selective LB plates for getting isolated colonies. After that step, colonies were inoculated in liquid selective LB medium and grown for 2 days. The transformant culture (3 ml) was centrifuged. Pellet was resuspended in 500 µl of CTAB Buffer and kept in a bath at 65 °C with gentle shaking for 1 hour. After that step, 250 µl of phenol and 250 µl of CI (24:1 chloroform:isoamylalcohol) were added. The mix was

shaken and centrifuged at 8900 g for 10 minutes. After that, the aqueous phase was collected and mixed with 500 µl of CI. Once homogenized, the suspension was centrifuged at 8900 g for 10 minutes again. The aqueous phase was newly collected, mixed with 750 µl of isopropanol (-20 °C) and kept at -20 °C for 20 minutes. After that, tubes were centrifuged at 8900 g and 4 °C for 10 minutes and pellet was washed with ethanol 70 % at -20 °C twice. After that step, pellet was centrifuged at 8900 g for a few seconds and kept opened on the flux cabinet in order to evaporate all the alcohol. Then, pellet was resuspended in 200 µl of TNE 1X, 3 ng of RNase (Sigma 31 µg/ml) were added and it was kept on a bath at 37 °C for 1 hour. In next step, 500 µl of CI were added and the mix was centrifuged at 8900 g for 10 minutes. The aqueous phase was collected and mixed with 500 µl of pure ethanol at -20 °C. It was kept at -20 °C for 30 minutes and, after this time, it was centrifuged at 8900 g for 10 minutes. The supernatant was discarded and pellet was kept opened on the cabinet again to remove all the ethanol. Once pellet was dry, it was resuspended in 50 µl of nuclease free water and kept on the fridge at 4 °C.

An electrophoresis with a 0.8 % agarose gel, as described in section 3.4, was run to check that the extraction was successful.

3.9. PCR with the extracted DNA for *B. firmus*

To check that the DNA belonged to *B. firmus* a PCR was done as the protocol described by Geng et al. (2016) [10], using the following specific primers: SEP1_F –:GCAATATGTTCGTGTAAT and SEP1_R: TGTTATCATCGTCTGTAA. These primers amplify the Sep1 gene. It's a nematicidal serine protease recently discovered in *Bacillus firmus*.

First of all the "master mix"* was prepared: 8 µl/tube of DreamTaq Green Buffer (PCR buffer), 1 µl/tube of dNTPs 10 mM (the free nucleotides), 1,2 µl/tube of primer forward 10µM (in this case the SEP1), 1,2 µl/tube of primer reverse 10 µM and 0,25 µl/tube of DreamTaq polymerase 5 U/µl.

*Master mix is how we call to the PCR mixture without the DNA sample. Preparing the master mix is a standard protocol to reduce pipetting errors, make the PCR mix more homogeneous between the different tubes and do it all in less time.

In this case the volumes were picked for 14 tubes: 10 samples, 2 positive controls (DNA from *B. firmus* "wild type", from the original bacterium stock), 1 negative control (without DNA) and 1 reaction extra for possible pipetting errors. Once the master mix was distributed in the tubes, 1 µl/tube of DNA was added. After that step, tubes were placed in a thermocycler (Bio Rad) and the following program was run: First the lid of the thermocycler was preheated to 94 °C. Next the mix was heated to 94 °C for 2 minutes. Then 40 cycles of (94 °C for 1 minute, 44 °C for 30 seconds and 72 °C for 30 seconds) were made and, finally, the mix was cooled to 4 °C for a long time to keep the PCR products cold until they were moved to the fridge.

Once the PCR was done an electrophoresis was run but this time with a 2 % agarose gel. This gel was more concentrated that the gel to check the quality of the DNA genomic or plasmid, because the PCR products are no longer than 300 pb. The rest of the protocol used was the same as described in section 3.4. The gel is also shown in the results and discussion section.

3.10. Plasmid stability assay and stock freezing

B. firmus GFP-Transformants were inoculated in selective LB plates and kept for 2 days at 35 °C. Isolated colonies were picked and inoculated again to repeat this process 4 times to reach a more stable plasmid. After that, *B. firmus* GPF-transformant colonies were grown for 2 days at 35 °C in selective LB liquid medium. After this time, the culture was dispensed in microcentrifuge tubes with a 15 % of glycerol and frozen in liquid nitrogen. The stock was kept at -80 °C.

3.11. Root colonization assay and sample preparation

Tomato seeds were surface-sterilized with 10 % NaClO for 1 minute, and then were washed three times with sterile distilled (1 min) (Bordallo et al., 2002) [3]. After that step, seeds were kept 24 hours in water at 4 °C. After that seeds were dried in sterile filter paper and were placed in sterile vermiculite (autoclaved and autoclaved again 24 h later). Seedling were watered and kept in a growth chamber for 20 days at 25 ºC and 16 h : 8 h (light : dark) photoperiod GFP-*B. firmus* transformants were grown as described previously for 3 days.

To study the *B. firmus*-plant interaction the experiment was carried out in a moist chamber. Briefly, a petri dish with sterile filter paper at the bottom was watered and an autoclaved slide was placed above the filter paper. A little segment of root (2 cm) was cut from the plant and placed on the slide. Next, 50 µl of the *B. firmus*-GFP culture were pipetted on the root surface. The petri dish was closed with parafilm and kept at 35 °C for 2 days.

To prepare the sample for live-cell imaging the root was watered to remove the *B. firmus* bacteria not adhered to the root surface and the root was placed in a new slide. After that, the cover was set over the root and pasted with nail polish to close it hermetically. At this point the root was ready to be visualized in a confocal laser microscope.

3.12. Nematode eggs infestation assay and sample preparation

50 nematode egg masses were extracted from tomato plants inoculated with *Meloidogyne javanica*. They were handpicked with the aid of tweezers and a binocular microscope. After that, egg masses were stirring with 1 ml of bleach 10 *% for 1 minute. Finally, 9 ml of distilled water were added to eliminate the bleach effect. At this point, the eggs concentration was calculated by counting the number of nematode eggs in a 10 µl of the suspension. The results are the mean of three replicate, and were obtain 6 eggs per µl, and approximately 2 of them in embryonic state. This is the most vulnerable state for *B. firmus* infestation.

Egg-infection bioassays were carried out as Escudero et al., (2016) [8]. Briefly, ten-well microscope slides (Waldemar Knittel) were used. Each well contained 25 µl (final volume) with approximately 10 surface-sterilized *Meloidogyne javanica* eggs, and 5 µl of *Bacillus firmus*-GFP transformant. The slides were maintained in a moist chamber for 2 days and wells without *B. firmus* were used as controls.

3.13. Confocal laser microscopy

The ideal conditions would have been to watch the root and the eggs 15 days and 72 hours after the inoculation respectively. But because of calendar reasons we only could make the photos after 48 hours.

The confocal laser microscope used was the LEICA TCS SP5 controlled by the Leica software. GFP florescence was detected at 505-530 nm and the natural fluorescence from eggs and root was detected at 580-620 nm in both cases. These wavelength ranges have been taken from a previous report (Escudero and Lopez-Llorca, 2012) [9]. The images taken were edited with ImageJ.

4. Results and discussion

First results are the experimental success:

According to the objectives, a very important result is the success in the transformation itself, as the achievement of all the previous steps as plasmid extraction and purification or protoplasts preparation.

However, the protoplasts transformation was not the first option. In first place we tried a heat shock transformation protocol with a previous preparation to make competent cells (cells able to be transformed by heat shock). Both protocols were from the Nick Talbot research group, (2017) [16], and they were used for other *Bacillus* species successfully. After some failures during 3 months, we changed to the protoplasts protocol described previously. It worked at the first try and with a high efficiency.

Next results are the electrophoresis gels:

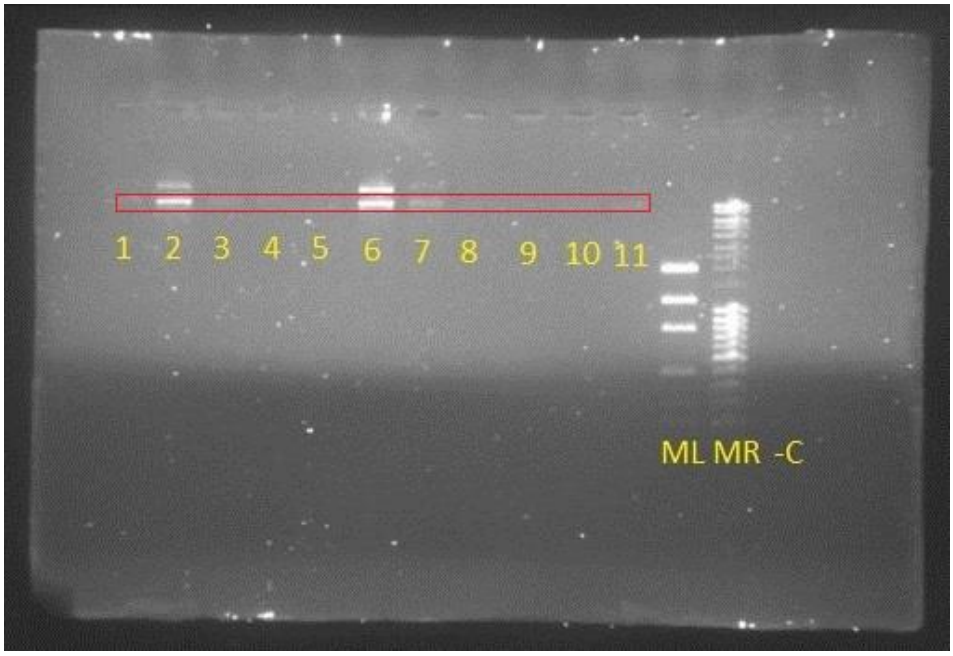

Figure 3: Electrophoresis gel with 11 plasmid samples after the extraction from *E. coli* and purification form the agarose gel. It also includes mass ruler, mass ladder and negative control.

As is shown in the Figure 3 most of the samples had little amounts of DNA. The amplicon of the marked row corresponds to the plasmid DNA amplicon so at this point we can consider the plasmid extraction and purification as successful.

Plasmids amounts from samples 2, 6 and 7 were the most concentrated ones and they were quantified with 9, 14 and 2 ng/µl respectively. The sample 6 was used for further transformations.

The cut of the plasmid row in the previous gel was done carefully in order to don't take rests of genomic DNA, placed in a row above the plasmid DNA. For this reason, the gel in the Figure 3 can't have genomic DNA and the row above our mark must be also plasmid DNA. Probably it's plasmid dimers.

Figure 4: Electrophoresis gel with 10 isolated colonies' DNA, mass ruler and controls.

In first place I have to apologize for the quality of this gel. The mass ruler is not extended and it's not possible to appreciate the amplicon of the marked row. This gel couldn't be repeated due to loss of the samples by experimental errors.

As is shown in the Figure 4 almost all the samples belonged to *B. firmus* but not the sample 2, where there wasn't amplification. Here is an evidence of the ubiquity of *Bacillus* and how important is taking in account all the controls carried out in the transformation process.

Actually in one of the previous heat shock transformations we found fluorescent *Bacillus* and, taking for sure that it was *B. firmus*, we spent 1 month working with the wrong bacterium before we did the DNA control.

The last results are the images from the confocal laser microscopy:

Regarding to the root colonization by *B. firmus,* some bacteria was adhered to the root surface but did not penetrate inside the root, and biofilm formation adhered to the roots was not appreciable after 48 h after inoculation at 35^{o}C (Figure 5).

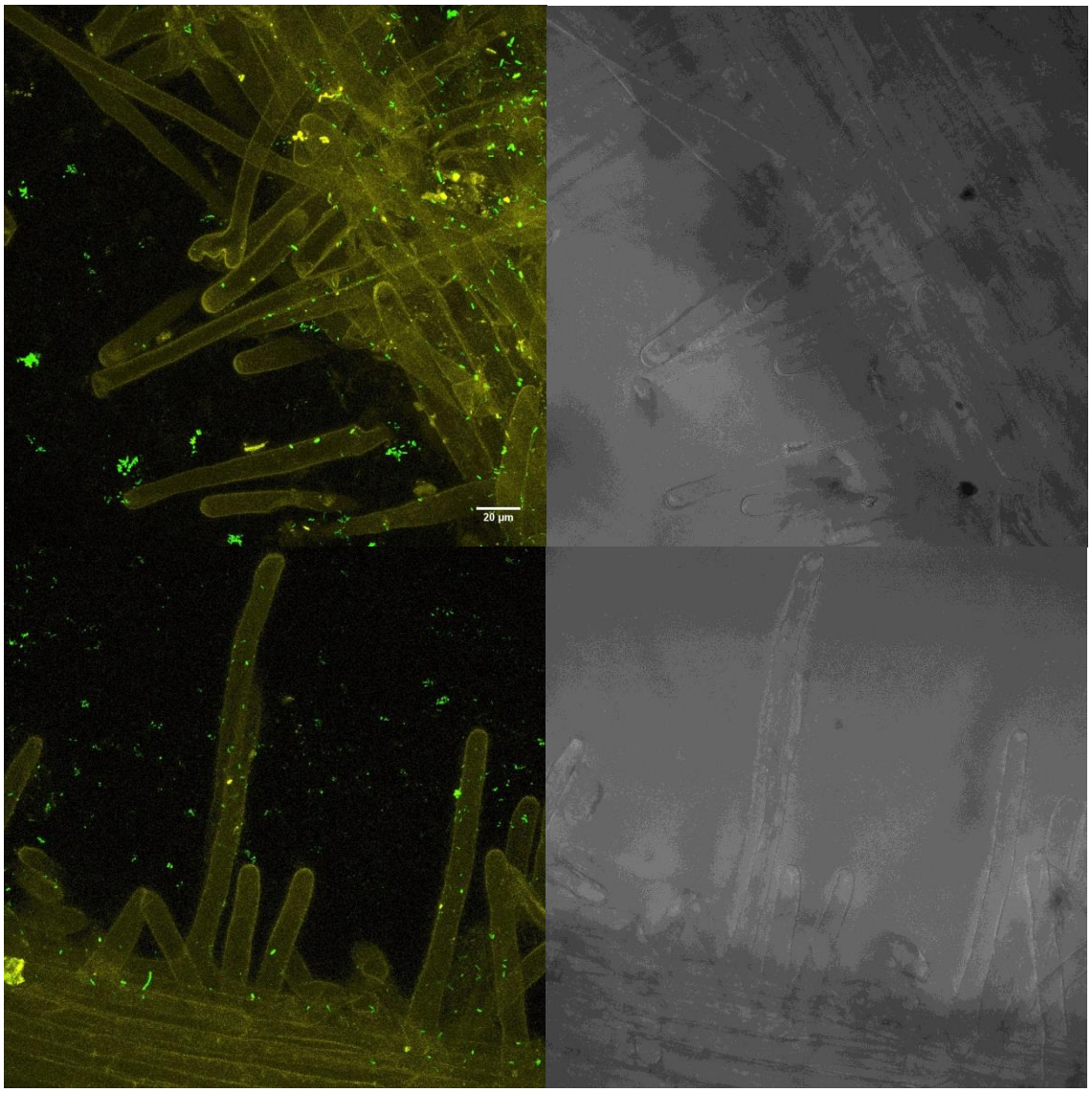

Figure 5: Tomato roots inoculated with the GFP transformant of *B. firmus* after 48h seen with laser confocal (left) and visible light (right). No endophytic colonization can be appreciated.

Some other *Bacillus* spp. are also able to develop on roots forming biofilms, such as *Bacillus subtilis* (Figure 6), but we can't see the biofilm formation in Figure 5 as clear as in Figure 6.

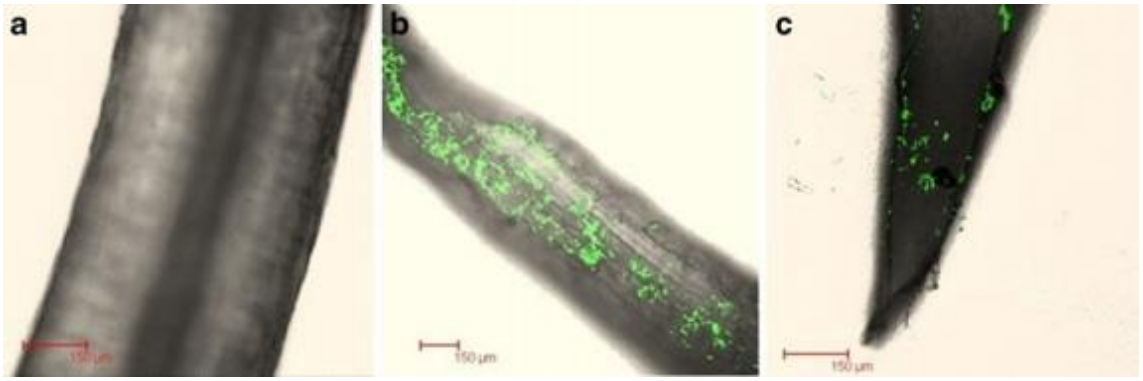

Figure 6: Micrographs of plant roots colonized by GFP-tagged *B. subtilis* and biofilm formation on cotton roots in a hydroponic system. "a" Control. "b" Bacterial biofilm formation on the elongation zone of a cotton root following incubation for 24 h. "c" Bacterial biofilm formation on a cotton root tip following incubation for 24 h (Li et al., 2013) [14].

In a previous experiment carried out by part of the UPC-Plant Pathology research group, *B. firmus* was isolated from inside a tomato root 50 days (at 25 °C) after the inoculation. This fact proves that the bacterium penetrates the root and colonizes the rhizosphere endophytically but probably it takes to the bacterium more than 48 hours. A longer incubation time also would probably allow the biofilm formation, and would induce active defense mechanisms against *Meloidogyne.*

The relationship between *B. firmus* and root-knot nematode eggs was also observed. The bacteria were able to develop on the egg surface forming biofilm, but did not penetrate inside the egg 48 h after inoculation and incubation at 35^{o}C (Figure 7). The entrance of the bacterial metabolites inside the egg could be a signal of the damage in the egg wall. This is probably a consequence of the nematicidal proteases produced by *Bacillus firmus,* as Sep1 (Geng et al. 2016) [10]. This fact would explain the hatching reduction by this bacterium observed in previous reports (Crow 2014 [5], Terefe et al. 2009 [18], Xiong et al. 2015 [24]).

In a previous assay we inoculated *Bacillus firmus* in nematode eggs as described in section 3.12 but incubating the bacterium for 3 days at 35°C. The eggs were observed with the microscope from Figure 2 and the embryonic eggs seemed to be almost destroyed (no photos were taken).

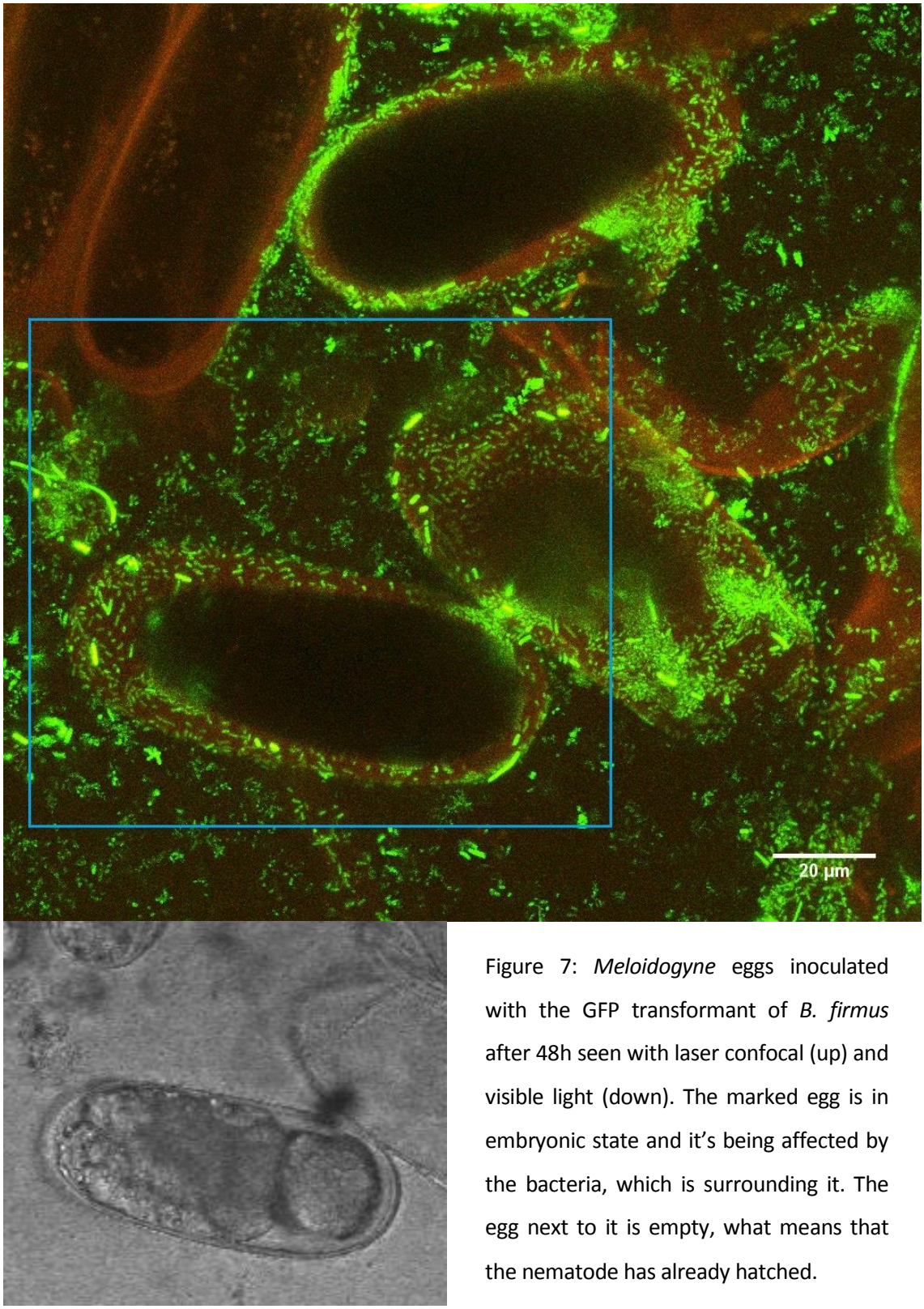

Figure 7: *Meloidogyne* eggs inoculated with the GFP transformant of *B. firmus* after 48h seen with laser confocal (up) and visible light (down). The marked egg is in embryonic state and it's being affected by the bacteria, which is surrounding it. The egg next to it is empty, what means that the nematode has already hatched.

5. Conclusions

The first conclusion is that protoplasts transformation is a more efficient protocol than heat shock transformation for *Bacillus firmus*.

We have shown that the protocols for plasmid extraction from *E. coli* and protoplast formation from *B. firmus* previously described were successful.

The ubiquity of the genus *Bacillus* also has been proved in this project.

Next conclusion is that 48 h after the inoculation roots don't show any symptom of colonization or biofilm formation because they need more time to be penetrated by *B. firmus*.

On the other hand, embryonic eggs seem to be seriously damaged. The bacteria appear to be adhered to its cuticle. Also it looks like the bacteria have not reached the egg inside although some of their cytoplasmic products are getting in due to the bad state of the egg wall. 24 more hours would be enough for the egg to be irreversibly destroyed. There is some biofilm formation.

In the future we recommend repeating this experiment with longer incubation times for the bacterium, especially on root samples.

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Annexed A

***Meloidogyne* life cycle and infection process** (Perry et al., 2009) [17]

Female lay eggs while produces a gelatinous matrix of glycoprotein. This compound protects the eggs from the environment and other organisms. The complex of eggs and the matrix is called egg mass. Inside the egg the embryo becomes a juvenile. Then, it moults to the second-stage juvenile (J2) which is the infective stage of the nematode.

After the hatching the J2 reaches for a plant root. Nematodes in the soil are vulnerable so it must find a host plant as soon as possible. Its lipid reserves keep it alive during this process. Once it has invaded the root it starts to develop a feeding site. Xylem and phloem protocells are induced to differentiate into specialized nurse cells which are called giant cells and it produces the root knot or gall. At this point the J2 becomes sedentary and fatter. After that, it moults to the third, fourth and adult stages. At the adult stage the female remains sedentary but not the male.

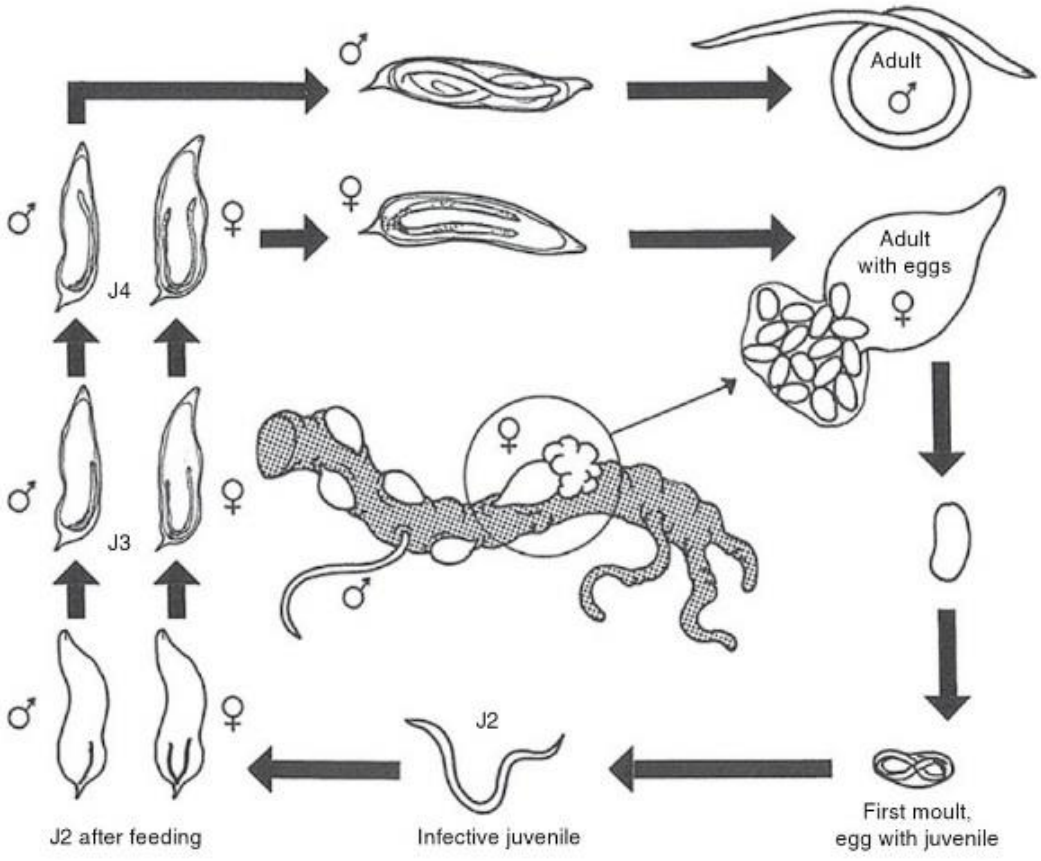


Figure 8: Life cycle of *Meloidogyne* (Perry et al., 2009) [12].

Annexed B

Description and growth conditions of *Bacillus firmus* (Vos et al., 2009) [23]

This bacterium is genetically heterogeneous and related to *Bacillus lenteus* and *Bacillus circulans*. Facultatively anaerobic, straight, round-ended, motile rods, 0.8-0.9 µm in diameter, that occur singly, in pairs or occasionally as short chains. Endosores are ellipsoidal or cylindrical, lie subterminally, paracentrally or centrally, and may swell the sporangia slightly. Colonies grown in TSA for 3 days at 30°C are 1-12 mm in diameter, creamy-yellow to pale orangey-brown in color. They are of butyrous consistency, have margins that vary from entire to finely rhizoidal and surface appearances that are egg-shell to glossy, sometimes with granulars or zoned areas in center. Temperature range for growth goes from 20°C to 50°C and the optimum lies between 30°C and 40°C. The pH range goes from 6 to 11.5 and the optimum is between 7 and 9. It grows on presence of 7% NaCl. Catalase-positive.

Figure 9: Streaked culture of *B. firmus* in LB medium.

Annexed C

Theoretical base of molecular methods used in this project

- Polymerase Chain Reaction (PCR)

The objective of this method is to make copies of a DNA segment. This is called to "amplify" the DNA. For this process we need the DNA sample, 2 primers (the forward and the reverse), the nucleotides to build the DNA, the Taq polymerase and other compounds to make a suitable buffer, like $MgCl_2$ which is an essential cofactor of the polymerase. This enzyme, the Taq polymerase, is an essential component of the reaction and it's a special polymerase able to work in high temperatures (around 65-72°C). All these products are mixed and submitted to high temperature cycles with a thermocycler*. The temperatures may change depending on the primers used for the experiment. Usually, there are 3 steps: First the temperature rises to around 95°C for the double strain DNA to become single strain DNA. At the second step the temperature decreases for the primers to get attached to the single strain DNA. Finally, at the third step the temperature is increased to reach an optimum range for the Taq polymerase to work. These steps are repeated over 40 times and the number of DNA segments is doubled in each cycle so this number increases exponentially. Normally after the PCR an electrophoresis is made to check if the DNA amplification was right.

*The thermocycler is a device designed only to make PCRs. Its work is to heat or cool the Eppendorf tubes where the reaction is taking place. It's programmable so you can choose the temperature and the duration time of every cycle.

Figure 10: Closed/Opened thermocycler where the PCRs where made in this project.

- Electrophoresis

This method is used to pull apart the molecules from a sample depending on their size and electrical charge. In this case we use it to divide DNA molecules, with negative charge, depending on their length. The electrophoresis takes place in a plastic bucket with electrodes. Inside of it there is a solid gel of agarose, which has to be made for every assay, covered by a liquid buffer. This gel is made with a mold and contains an intercalating agent (ethidium bromide, for example) that is attached to the DNA during the electrophoresis. Also the gel can be made with more or less agarose concentration according to the length of the DNA fragments we want to load. The DNA sample, mixed with a loading buffer, is loaded inside de gel and then the bucket is electrified. The electric camp makes the DNA move through the gel to the positive electric pole, placed at the other side of the gel. After a few minutes the gel is taken out from the bucket and analyzed with UV light, which makes the ethidium bromide shine. This way we can see the DNA and its distribution across the gel. Normally it's also loaded a marker like a mass ruler (DNA fragments of different lengths in different concentrations) to compare with our sample.

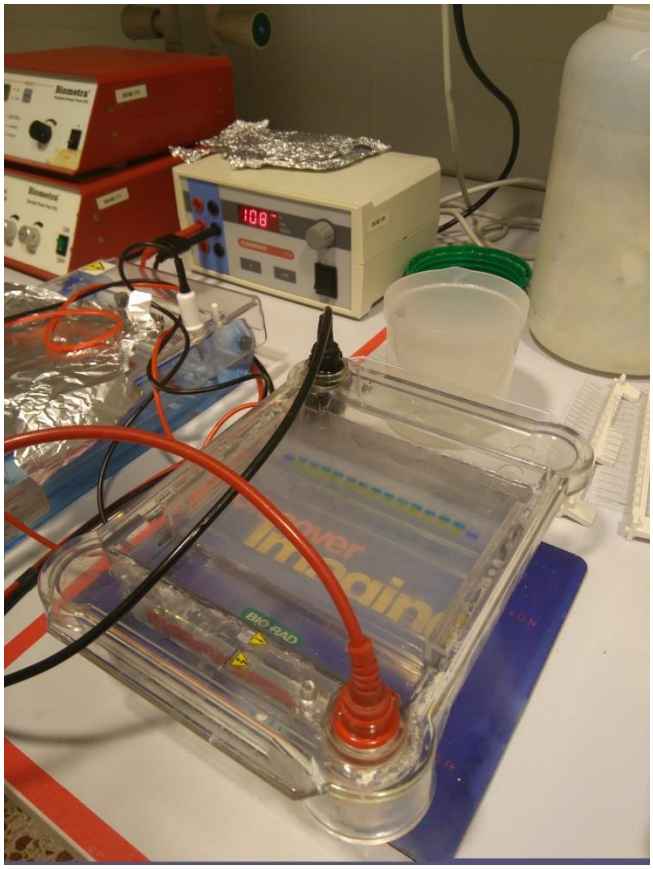


Figure 11: Electrophoresis in process. All the electrophoresis run in this project were made with this stuff.

- Genetic transformation

Transformation is the process of insert a DNA plasmid in a unicellular organism.

A plasmid is a circular fragment of double strain DNA. It may content the gene or genes that we want to add to our organism. Usually it also contains at least one gene for one antibiotic resistance. This is because after the transformation the cells are spread on selective medium (medium with antibiotic) so only the transformed cells survive. It's like a selection filter.

There are some different protocols for genetic transformation and not all of them work in all the organisms. Some examples are the heat-shock or the electroshock. These shocks produce temporal holes in the cells membrane where the plasmid can go through. Both protocols need the cells to be previously prepared. After that they are called "competent" cells. This competence consists in a calcium bath that makes the plasmid get pasted in the cellular membrane before the transformation. In this project the protocol used was the transformation by protoplast formation. The main idea of this method is to destroy the cellular wall without breaking the membrane of the cells we want to transform. This way they become protoplasts and the plasmid can get inside the cell more easily during the process.

Once the plasmid is inside the cell it can start to be read and duplicated by the host cell. The plasmid will stay in the cytoplasm and will not get mixed with the genomic DNA from the organism. The cell could delay some time to read the plasmid and synthetize its proteins so after the transformation it's necessary to wait for 1 or 2 hours before put the cells in antibiotic. Another fact to take in count is that the plasmid is not always duplicated. This means that the transformants must stay in selective medium and it's recommendable to streak the cells in plates to isolate a single colony for 3 or 4 times. This way the plasmid in the final cells is more "stable".

Annexed D

Plasmid composition and origin (Dunn and Handelsman, 1999 [7]; BGSC, 2013 – 2017 [1])

pAD43-25 is a shuttle vector which can be replicated in *E. coli* and *Bacillus*. It contains a promoter from *Bacillus cereus* attached to the GFP gene. This promoter allows the GFP expression in *Bacillus*. This plasmid was made with a promoter trap, pAD123, which was constructed with fragments of three other plasmids: pKK232-8 (containing the ampicillin resistance and the replication origin for *E. coli*), pHP13 (containing the chloramphenicol resistance and the replication origin for *Bacillus subtilis*) and pFV25 (containing the GFP gene). pAD123 was used to build a library of this plasmid combined with a lot of different random fragments of the B*acillus cereus* genome. One of the successful ones was pAD43-25 which trapped the upp gene promoter. The features of this plasmid are:

- gfpmut3a: Promoter-less gene
- encoding a variant of green fluorescent protein.
- rep: Replication initiation protein from *Bacillus subtilis*.
- cat: Encodes chloramphenicol acetyl transferase (Cm resistance) in both *E. coli* and B.Subtilis.
- bla: Encodes β-lactamase (ampicillin resistance) in *E. coli* only.
- ‘glyA: Last 1010 bp of the *Bacillus cereus* structural gene for jlycine/serine hydroxymethyltransferase.
- P_{upp}: Constitutive promoter from the *Bacillus cereus* upp gene (uracil phosphoribosyltransferasa).
- Some restriction targets.

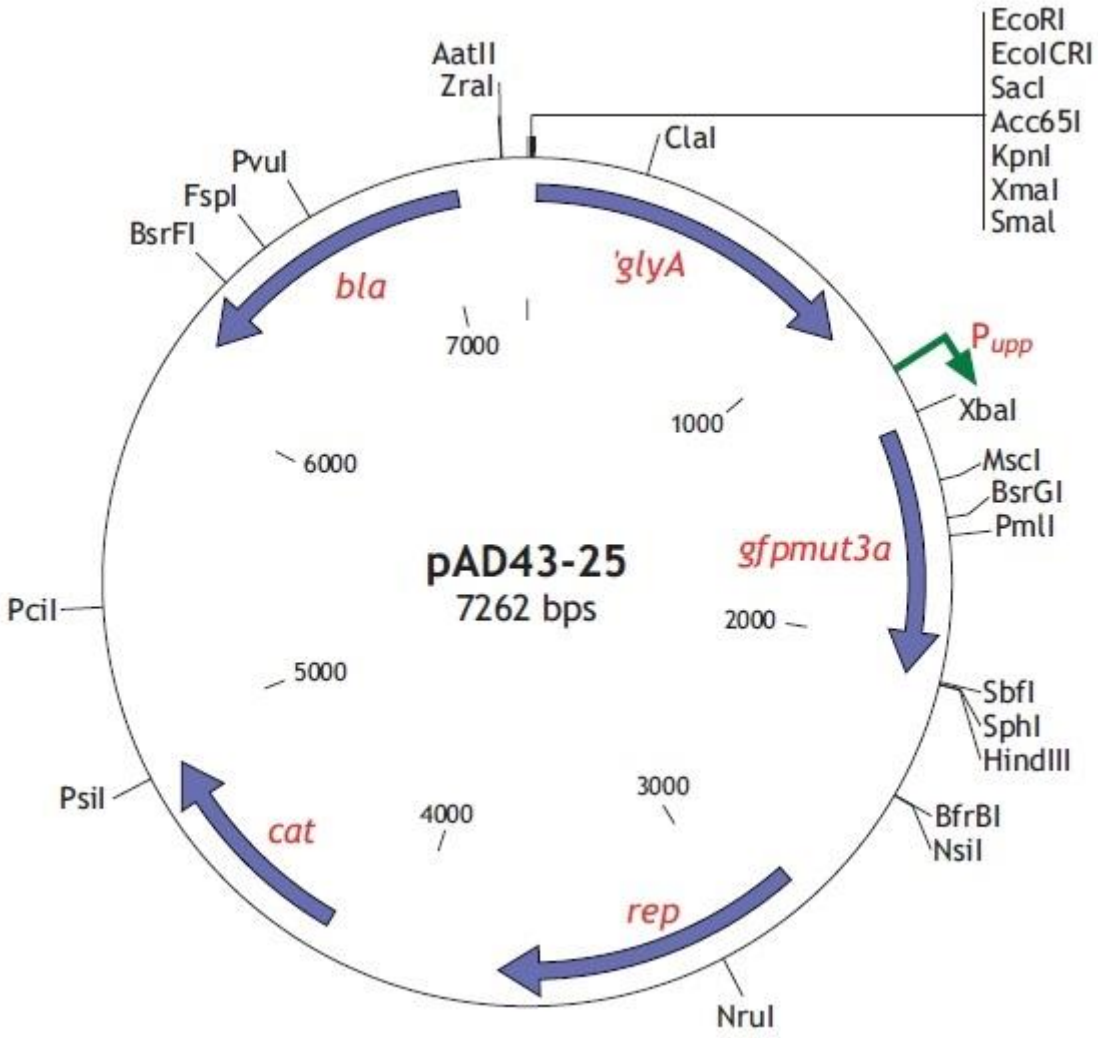


Figure 12: pAD43-25 scheme with the position of all the features. (BGSC 2013 – 2017) [1].

Annexed E

Mass Ruler, Mass Ladder, DreamTaq Green Buffer and NucleoSpin PCR clean-up manufacturer details and protocols (Thermofisher [21][19][20], Macherey-Nagel [15] respectively)

thermo scientific

PRODUCT INFORMATION

Thermo Scientific MassRuler DNA Ladder Mix, ready-to-use

Pub. No. MAN0013021
Rev. Date 9 December 2016 (Rev. B.00)

Lot: _ **Expiry Date:** _

Components	#SM0403
MassRuler™ DNA Ladder Mix, ready-to-use, 103 ng/µL	2 × 500 µL (for 50-200 applications)
6X MassRuler™ DNA Loading Dye	1 mL

Store: at room temperature or at 4°C for periods up to 6 months. For longer periods store at -20°C.

www.thermofisher.com

For Research Use Only. Not for use in diagnostic procedures.

Description

Thermo Scientific™ MassRuler™ DNA Ladder Mix, ready-to-use, is designed for fast and accurate quantification and sizing of DNA fragments on agarose gels. The ladder contains the following 20 discrete fragments (in base pairs): 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80.

The ladder is ready to use – it is premixed with MassRuler™ DNA Loading Dye and can be directly applied onto an agarose gel.

Storage and Loading Buffer

10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.005% bromophenol blue and 10% glycerol.

6X MassRuler DNA Loading Dye

10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 60% glycerol and 60 mM EDTA.

CERTIFICATE OF ANALYSIS

Well-defined bands are formed during agarose gel electrophoresis.

The absence of nucleases is confirmed by a direct nuclease activity assay.

Quality authorized by: Jurgita Zilinskiene

Rev.9 53

Protocol for Loading

Step 1: Mix gently
Step 2: Load 5-20 µL per gel lane.

Recommendations

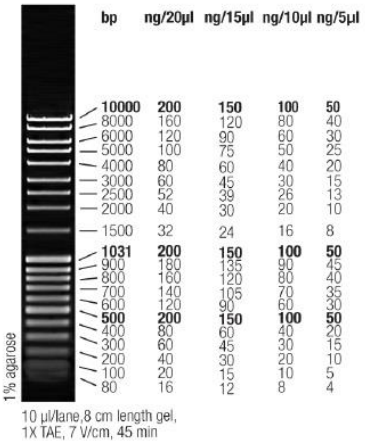
- Do not heat before loading.
- For accurate DNA quantification:
  - dilute your DNA sample with the 6X MassRuler DNA Loading Dye (#R0621, supplied with the ladder) Mix 1 volume of the dye solution with 5 volumes of the DNA sample.
  - load the same volumes of the DNA sample and the DNA ladder;
  - adjust the concentration of the sample such that the expected amount of DNA loaded is approximately equal to that of ladder's band of a nearest size.
- For DNA band visualization with SYBR™ Green and other intercalating dyes, do not add the dyes into the sample, use gel staining after electrophoresis or include dyes into agarose gel to avoid aberrant DNA migration.

Important note

- For DNA bands visualization with GelRed™ use gel staining after electrophoresis to avoid aberrant DNA migration.

Note. The apparent intensity of bands containing equal ng quantities of DNA may differ in different horizontal sections of gel (diminishes from top to bottom).

MassRuler DNA Ladder Mix, ready-to-use



bp	ng/20µl	ng/15µl	ng/10µl	ng/5µl
10000	**200**	**150**	**100**	**50**
8000	160	120	80	40
6000	120	90	60	30
5000	100	75	50	25
4000	80	60	40	20
3000	60	45	30	15
2500	52	39	26	13
2000	40	30	20	10
1500	32	24	16	8
1031	**200**	**150**	**100**	**50**
900	180	135	90	45
800	160	120	80	40
700	140	105	70	35
600	120	90	60	30
500	**200**	**150**	**100**	**50**
400	80	60	40	20
300	60	45	30	15
200	40	30	20	10
100	20	15	10	5
80	16	12	8	4

10 µl/lane,8 cm length gel,
1X TAE, 7 V/cm, 45 min

invitrogen
by life technologies

Low DNA Mass Ladder

Cat. no. 10068-013 Size 200 µL Store at –30°C to –10°C

Doc. Part no. 10068013.pps Pub. no. MAN0000782 Rev. 2.0

Description

The Low DNA Mass Ladder is composed of an equimolar mixture of six blunt-ended DNA fragments of 2000, 1200, 800, 400, 200 and 100 bp. Electrophoresis of 4 µL of the Low DNA Mass Ladder results in bands containing 200, 120, 80, 40, 20, and 10 ng of DNA, respectively.

Note: The Low DNA Mass Ladder does not contain loading buffer. Add gel loading buffer to the ladder as described in the following sections and see the table on page 2 for the amount of DNA per band in a given volume of ladder.

Storage Buffer

10 mM Tris-HC1 (pH 7.5), 1 mM EDTA

Prepare the Ladder with Loading Dye

Mix four volumes of Low DNA Mass Ladder with one volume of gel loading buffer containing dye (e.g., 4 µL ladder with 1 µL dye). Then load the ladder/dye mixture on the gel.

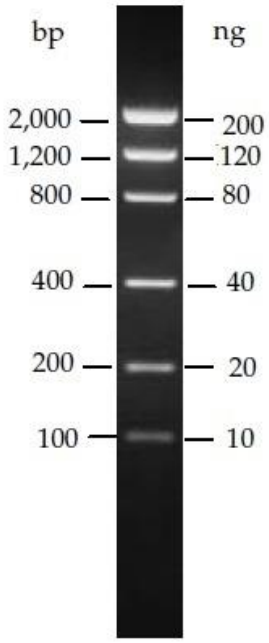
The table on page 2 shows the appropriate volume of ladder to use to estimate the mass of unknown DNA samples. For a reliable comparison of band intensities, make sure the loading volume of the ladder is the same as the volume of the experimental sample. Smaller volumes routinely give sharper bands. *Do not heat the ladder before loading.*

Note: The closer the size of the sample band relative to the band of comparable intensity in the Low DNA Mass Ladder, the more accurate the mass estimation will be.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

For research use only. Not for use in diagnostic procedures.



4 µL/lane; 2% agarose gel; stained with ethidium bromide

Amount of DNA (ng) per Band

The following table shows the amount of each DNA fragment in a given volume of ladder.

IMPORTANT! The volumes in the table are for ladder only, not ladder plus dye. For example, if you loaded 4 µL of ladder with 1 µL of dye, use the values in the **4 µL** column.

Volume of Low DNA Mass Ladder			
Fragment size	2 µL	4 µL	8 µL
2000 bp	100 ng	200 ng	400 ng
1200 bp	60 ng	120 ng	240 ng
800 bp	40 ng	80 ng	160 ng
400 bp	20 ng	40 ng	80 ng
200 bp	10 ng	20 ng	40 ng
100 bp	5 ng	10 ng	20 ng

Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific 10X DreamTaq Green Buffer

#B71 4 x 1.25 mL

Lot: __

Expiry Date:

Store at -20°C

Description

10X Thermo Scientific™ DreamTaq™ Green Buffer is a proprietary PCR buffer which contains KCl and $(NH_4)_2SO_4$ at a ratio optimized for robust performance of DreamTaq™ DNA Polymerase in PCR and includes $MgCl_2$ at a concentration of 20 mM. In addition the buffer includes a density reagent and two tracking dyes. The density reagent allows direct loading of PCR products on a gel. The blue dye (migrates with 3-5 kb DNA fragments in 1% agarose gel) and the yellow dye (migrates faster than 10 bp DNA fragments in 1% agarose gel) are included for monitoring electrophoresis progress. The dyes have excitation peaks at 424 nm and 615 nm.
For applications that require PCR product analysis by absorbance or fluorescence excitation, we recommend using the colorless 10X DreamTaq Buffer (#B65) or purifying the PCR product using the GeneJET™ PCR Purification Kit (#K0661) prior to analysis.

CERTIFICATE OF ANALYSIS

Tested for the absence of endo-, exodeoxyribonucleases, ribonucleases and functionally tested in amplification of 956 bp DNA fragment of a single-copy gene of human genomic DNA.

Quality authorized by: Jurgita Zilinskiene

PRODUCT USE LIMITATION

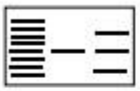
This product is developed, designed and sold exclusively *for research purposes and in vitro use only*. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.
Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.

NucleoSpin® Gel and PCR Clean-up is developed as a 2-in-1 kit allowing DNA fragments to be purified from enzymatic reactions, such as PCR, as well as from agarose gels.

The sample is mixed with Binding Buffer NTI and in case of a cut-out gel band, it is heated to dissolve the agarose. In the presence of chaotropic salt, the DNA is bound to the silica membrane of a NucleoSpin® Gel and PCR Clean-up Column. Contaminations are removed by simple washing steps with ethanolic Wash Buffer NT3. Finally, the pure DNA is eluted under low salt conditions with slightly alkaline Elution Buffer NE (5 mM Tris/HCl, pH 8.5).

PCR clean-up, gel extraction

Protocol-at-a-glance (Rev. 04)

	PCR clean-up	Gel extraction	DNA clean-up (with SDS)	Single stranded DNA clean-up
1 **PCR clean-up, DNA clean-up, or single stranded DNA clean-up:** Adjust binding condition **Gel extraction:** Excise DNA fragment / solubilize gel slice	 200 µL NTI/ 100 µL PCR	  200 µL NTI/ 100 mg gel 50 °C 5–10 min	 500 µL NTB/ 100 µL sample	 200 µL NTC/ 100 µL sample
2 **Bind DNA**			11,000 x *g* 30 s	
3 **Wash silica membrane**			700 µL NT3 11,000 x *g* 30 s Recommended: 2nd wash 700 µL NT3 11,000 x *g* 30 s	
4 **Dry silica membrane**			11,000 x *g* 1 min	
5 **Elute DNA**			15–30 µL NE RT 1 min 11,000 x *g* 1 min	